Structural evidence of quercetin multi-target bioactivity: a reverse virtual screening strategy

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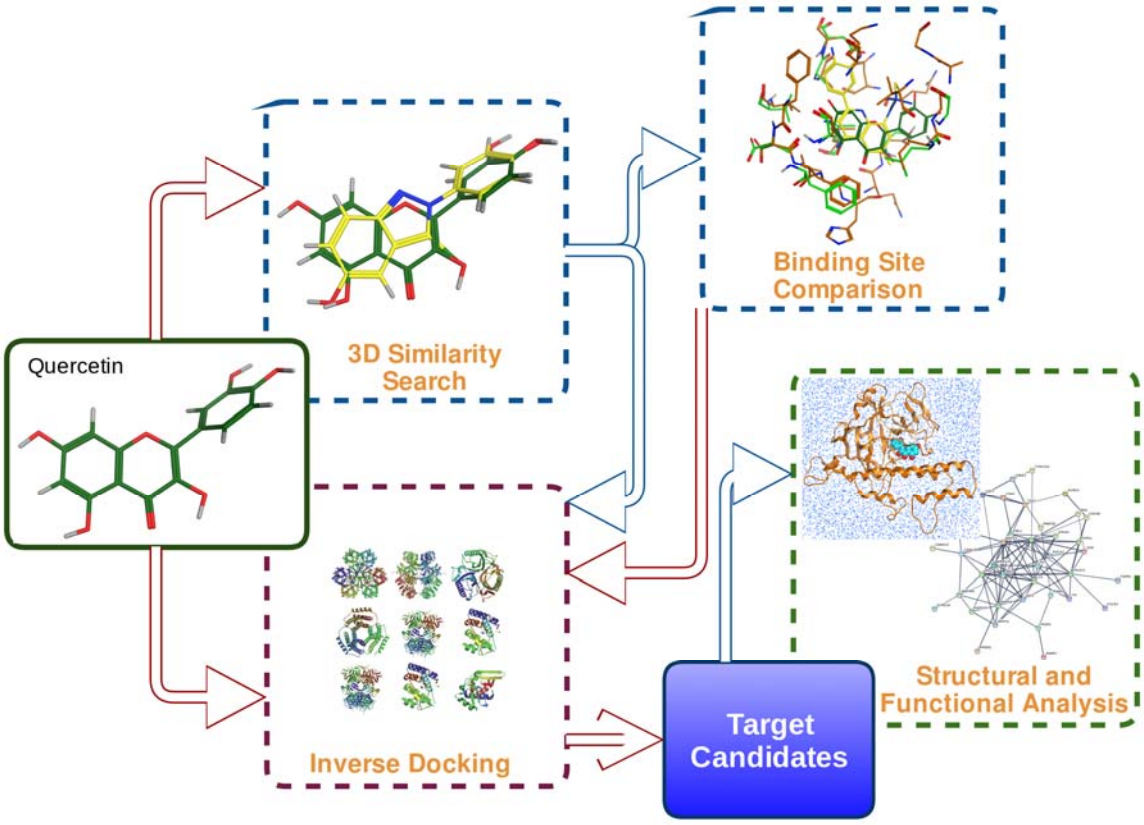
ABSTRACT

The ubiquitous flavonoid quercetin is broadly recognized for showing diverse biological and health-promoting effects, such as anti-cancer, anti-inflammatory and cytoprotective activities. The therapeutic potential of quercetin and similar compounds for preventing such diverse oxidative stress-related pathologies has been generally attributed to their direct antioxidant properties. Nevertheless, accumulated evidence indicates that quercetin is also able to interact with multiple cellular targets influencing the activity of diverse signaling pathways. Even though there are a number of well-established protein targets such as phosphatidylinositol 3 kinase and xanthine oxidase, there remains a lack of a comprehensive knowledge of the potential mechanisms of action of quercetin and its target space. In the present work we adopted a reverse screening strategy based on ligand similarity (SHAFTS) and target structure (idTarget, LIBRA) resulting in a set of predicted protein target candidates. Furthermore, using this method we corroborated a broad array of

previously experimentally tested candidates among the predicted targets, supporting the suitability of this screening approach. Notably, all of the predicted target candidates belonged to two main protein families, protein kinases and poly [ADP-ribose] polymerases. They also included key proteins involved at different points within the same signaling pathways or within interconnected signaling pathways, supporting a pleiotropic, multilevel and potentially synergistic mechanism of action of quercetin. In this context we highlight the value of quercetin's broad target profile for its therapeutic potential in diseases like inflammation, neurodegeneration and cancer.

Keywords: drug target prediction; molecular docking; flavonoids; quercetin.

Graphical abstract



1. Introduction

Phytochemical rich diets are correlated with increased longevity and a wide range of health benefits including a decreased incidence of cardiovascular diseases and a slowed progression of cerebrovascular diseases (Commenges et al., 2000; McCullough et al., 2012). Flavonoids constitute one of the largest families of phytochemicals found in plants. Alongside their contribution to the flavor and the color of fruits and vegetables, flavonoids are widely recognized for their direct antioxidant properties (Cao et al., 1997; Fuhrman and Aviram, 2001). In particular, many flavonoids display anti-inflammatory, anti-carcinogenic, and neuroprotective effects among other bioactivities (Arredondo et al., 2010; Spencer 2012; Vidya Priyadarsini et al., 2010).

This ubiquitous polyphenolic group comprises several subclasses such as flavones (including flavonols), flavanones, flavans (including flavanols), isoflavones, chalcones and anthocyanidins. Flavonols are by far the most abundant and widely distributed in nature, with quercetin (*2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxychromen-4-one*) being a prototypical flavonol. Despite having well characterized antioxidant actions, there is an emerging view that the neuroprotective, anti-inflammatory and anti-cancer effects of flavonoids are likely mediated by interaction with specific proteins involved in intracellular signaling cascades (Spencer, 2009; Williams et al., 2004). Currently quercetin and related flavonoids are well known phosphatidylinositol 3 kinase (PI3-kinase), xanthine oxidase and cyclooxygenase inhibitors but beyond these targets, a complete understanding of the underlying mechanism of action of quercetin requires the description of its whole target space. Modulating multiple targets is potentially more beneficial than a single target in terms of overall efficacy, possible side effects, resistance or compensatory mechanisms andsuch a multi-modal mechanism of action for quercetin supports its therapeutic potential (Koeberle and Werz, 2014).

While multi-target detection by wet-lab studies such as affinity chromatography, expression cloning and protein microarray is work- and time-intensive, *in silico* target prediction is a valuable

inexpensive and complementary approach that could bring an unbiased view of the potential protein targets of quercetin (Hart, 2005). ReverseScreen3D, INVDOCK, Tarfisdock, PharmMapper and idTarget are some of the *in silico* target prediction tools available (Cereto-Massagué et al., 2015; Koutsoukas et al., 2011). Employing these platforms has led to the identification of protein targets for a number of novel bioactive compounds such xyloketal and kinetin as well as for approved drugs like mebendazol (Dakshanamurthy et al., 2012; Kumar et al., 2015; Su et al., 2014). For a comprehensive review on the topic please refer to (Cereto-Massagué et al., 2015).

In the present study we propose a hierarchical screening of quercetin protein targets based on the sequential arrangement of ligand similarity search, binding site comparison and inverse docking. Additionally, a selected set of targets was assessed by means of molecular dynamics simulations demonstrating favorable binding with quercetin. Taking together, these approaches resulted in a predicted set of protein target candidates that can be related to the biological effects of quercetin. Supporting these findings, a broad array of previously proposed quercetin protein targets were identified as positive hits in this screening approach validating its utility. Above all, through its ADP/ATP mimetic capacity, quercetin might influence several proteins from related signaling pathways implying a potentially multilevel and synergistic mechanism of action.

2. Methods

2.1 Reverse Screening

The current *in silico* strategies for target identification include ligand-based, structure-based, machine learning-based and biochemical network-based approaches. When searching for potential target proteins in the first case, a query ligand is compared against a library of known interacting compounds and protein targets are retrieved by association (Klabunde, 2007). Alternatively, binding site comparison relies on the structure similarity between known and unknown protein targets. Inverse docking is another structure-based method, contrary to classic docking schemes it screens and ranks a protein library instead of a ligand library.

The present work followed a hierarchical screening scheme, which took advantage of the fastest methods (ligand similarity and binding site comparison) in its first stages to reduce the search space for the most expensive approach (docking) and to reduce the number of false positives obtained (Kumar and Zhang, 2015). For each step in the screening we chose three of the most recent publicly available software's for *in silico* target prediction (Fig. 1), also including protein candidates with similar binding site recognized by our in-house-developed software LIBRA (Ligand Binding Site Recognition Application) (Hung et al., 2015).

2.1.1 The ligand-target database

A database of suitable ligands contained in the Protein Data Bank was developed for ligand-based screening (Berman, 2000). Firstly, all the ligands contained in the PDB were downloaded from the 2014 version of *LigandExpo database*, a repository for co-crystalized structures and hetero-atoms in general indexed with the corresponding protein entry (Feng et al., 2004). The molecular structures were downloaded in SMILES-Stereo format as a whole database and rebuilt in Molecular Operating Environment (MOE) software (Chemical Computing Group Inc., 1010 Sherbooke St. West, Suite #910, Montreal, QC, Canada, 2011). Starting from 17969 PDB ligand entries, a series of hierarchical filters were applied with MOE to construct the database as follows: 1) Hydrogenation was corrected; 2) Undesirable small molecular weight ligands (salts and water molecules) and repeated entries were removed; 3) Molecules below the molecular weight of the flavonoid frame were deleted and Oprea rules were applied to restrict the database to flavonoid-like molecules and drug-like molecules respectively. Oprea rules are a refined version of the classical Lipinski's rules of five to determine drug likeliness (Oprea et al., 2007); 4) Molecules containing Se or As were also deleted. The current molecular database was energy minimized in MOE with a Merck molecular force field (MMFF94x) until 0.01 kcal/mol RMS gradient and saved in mol2 format. The final database contained 9924 different molecules.

2.1.2 Ligand-based screening of quercetin

Taking quercetin as a probe, we searched our in-house ligand-target database for similar co-crystallized ligands in the PDB. Quercetin molecular structure was built in MOE, energy minimized (MMFF94x, 0.01 kcal/mol RMS gradient) and saved in mol2 format for screening processes. SHAFTS (SHApe-Feature Similarity) algorithm implemented in its stand-alone version was used to screen quercetin against the conformers obtained from the ligand-target database. SHAFTS is an algorithm for tridimensional structural similarity calculation and ligand-based virtual screening (Liu et al., 2011). SHAFTS has been validated with decoy benchmark datasets and has demonstrated satisfactory active compounds enrichment and scaffold hopping capability against several representative kinases in retrospective virtual screening studies (Bai et al., 2012; Kong et al., 2012; Liu et al., 2011; Lu et al., 2011). It adopts a similarity metrics considering both molecular shape and pharmacophoric characteristics (hydrophobic center, positive or negative charge center, hydrogen bond acceptor and donor, and aromatic rings). A feature triplet hashing method is used for the fast rigid alignment of molecular structure. It finally returns a sorted list of molecule identifiers associated with structural similarity score against the query and the corresponding structural alignment.

Because SHAFTS uses a semi-rigid strategy for structural alignment, the conformational analysis program Cindy was used to generate a maximum of 100 lowest-energy conformers for each molecule in the ligand-target database (Liu et al., 2009). SHAFTS was then implemented with its default configuration. Protein complex identifiers were retrieved for ligands with a similarity score against quercetin above 1.5 (maximum 2.0) and subjected to the next stage of the screening.

2.1.3 Target candidate amplification based on binding site similarity

Delineating binding site similarities for proteins is another possible route for finding new targets for existing ligands. The protein candidates selected by SHAFTS were subjected to binding site comparison against a representative set of proteins contained in the PDB using our in-house-developed software LIBRA[RW1] (Hung et al., 2015). LIBRA is a tool for searching local structural

similarities between a protein structure and a collection of functional sites and their environment. It employs a graph-based approach in conjunction with a database of more than 170 thousand ligand binding sites generated by extracting residues surrounding the ligand from approximately 75 thousand structures of protein-ligand complexes deposited in PDB. Similar sites were defined according to these requirements: 100 % of similar residues (blosum62 scoring), minimum motive size of 5 residues, maximum structural alignment RMSD of 1 Å (Ångström) and no steric clashes between the ligand from the query protein and the known protein.

2.1.4 Structure-based screening

The optimized structure of quercetin was uploaded onto the online inverse dock server idTarget for screening against the selected list of protein candidates obtained in the previous stage.

idTarget performs inverse molecular docking for a query molecule against the whole PDB or a custom list of protein structures (Wang et al., 2012). It applies a divide and conquer algorithm to search the protein surface for suitable docking sites and a state-of-the-art scoring function optimized for binding energy prediction and evaluated with external decoy sets (Wang et al., 2012, 2011). A protein target *i* for a ligand *j* is selected considering the affinity profile for the ligand. Analogously to the use of docking decoys a Z-score is calculated according to: $Z_{ij}=(E_{ij}–E_i)/sd_i$, where E_{ij} is the dock score of ligand *j* to the protein pocket *i*, E_i and sd_i are the center and width of the affinity profile of protein *i*.

We selected the server scanning mode as it is the most exhaustive, where usual molecular docking procedures are carried out for each protein structure. Proteins were considered to be possible ligand targets if the idTarget binding energy was lower than -9.0 kcal/mol (250 nM predicted affinity) with a Z-score of 1.0 or lower (adapted from (Nikolić et al., 2015)).

In order to assess the reliability of the results, docking studies were also performed using MOE against target candidates. Protein candidate crystallographic structures were retrieved from the PDB as its biological unit. Each structure was optimized before analysis, hydrogen atoms were adjusted

and charges assigned with Amber12 force field, missing loops and atoms were completed by structure preparation module. For docking purpose, binding sites were defined by the corresponding co-crystallized ligand, and co-factors were included as part of the receptor if present. Default docking parameters were used such as Triangle Matcher Algorithm with London dG (scoring) and GBVI/WSA-dG (re-scoring) functions to generate 10 poses of each ligand. It should be taken into account that for the docking strategy, the solvent was not considered explicitly but using a scoring function in which the desolvation was calculated in an empirical way.

2.2 Molecular dynamics simulations of quercetin-target candidate complexes

Molecular dynamics (MD) simulations used the Nose Poincare Anderson (NPA) method implemented in MOE. The constant number of particles, volume, and temperature (NVT) ensemble with T) 300 K was used. The complex was immersed in a periodic water box with 6 Å margin with neutralizing ions (NaCl), and the whole system minimized to 1 kcal/mol RMS gradient before starting the MD run. Prior to the main simulation of 10 ns, a 100 ps stage gradually heated the molecular system to 300 K. Between the heating stage and main simulation the system velocities were reassigned which can improve sampling (Su et al., 2015). The interaction potential energy between the atoms of the ligand and the rest of the system was registered along the simulation and quercetin interactions were assessed by MOE PLIF module with default parameters. Equilibration was monitored by convergence in terms of the temperature, energy, and the RMSD (root-mean-squared deviations) of the backbone atoms as compared to the crystal structure of both complexes.

2.3 Functional annotation of protein target candidates

The final set of protein candidates was annotated according to protein structure and family, activity and involvement in disease. The corresponding human homologue was taken for proteins belonging to other mammals. The information was obtained from different sources including publicly available databases and tools like PDB, Uniprot (Consortium, 2014), DAVID (gene ontology terms and literature information) (Dennis et al., 2003), KEGG (biological pathways) (Kanehisa and Goto,

2000), GO TermMapper (gene ontology terms) (Boyle et al., 2004; Harris et al., 2004) and FlyMine (gene ontology terms and literature information) (Lyne et al., 2007) which bring complementary data. In addition, molecular targets identified by different approaches can be mapped onto specific disease-associated networks or pathways and target databases to construct protein-protein interaction networks. The "Search Tool for the Retrieval of Interacting Genes/Proteins" (STRING) database integrates information about interactions of proteins from different types of databases and was used for this purpose (Kuhn et al., 2012).

3. Results and discussion

3.1 Reverse screening

3.1.1 Ligand-based screening of quercetin

Using quercetin as an input molecule for SHAFTS algorithm we screened a database of pharmacologically relevant ligands associated with protein complexes in the PDB. Quercetin-like compounds were selected based on SHAFTS similarity score obtaining a list of 34 molecules which share the typical flavonoid scaffold with the exception of those identified by PDB as FL9, PIT, RE2, SLX, STL and DEH (see Table 1 from supplementary material). The corresponding co-crystallized protein set comprised 25 mammalian proteins. Proteins from plants and other organisms were not considered for the subsequent analysis.

3.1.2 Binding site comparison

Based on LIBRA analysis on local similarity at the protein binding site we conducted an amplification stage of the candidates found by ligand-based screening. Interestingly, most of the proteins identified also bind ligands with some similarity to quercetin and can be found in the first screening stage with a SHAFTS score ranging from 1.0 to 1.3 (see Table 2 from supplementary material). Such consistency between both approaches suggests that additional reliable candidates could have been initially found with a less stringent cut-off ligand based screening.

Along with the previous set of candidates three main protein classes can be distinguished,

sulfotransferases, tankyrases-poly(ADP-ribosyl)transferases and kinases. Most of these kinases share the same fold according to PFAM, the protein kinase domain (PF00069) followed by the tyrosine kinase domain (PF07714). Another kinase domain present in the candidate dataset is the PI3-kinase domain (PF00613) corresponding to the well-known quercetin target phosphatidylinositol 3 kinase (Walker et al., 2000).

3.1.3 Structure-based screening

From the previous screening stages, a list of 74 protein target candidates was identified. This set of proteins was then subjected to structure-based screening against quercetin using idTarget. This procedure allowed us to obtain protein target candidates associated with docking poses for quercetin and ranked according to their predicted affinity (see Tables 3 and 4 from supplementary material). The predicted affinity ranges from 15.1 nM to 494.1 nM, and has minor variations depending on the specific crystal structure for a given protein. From the unranked set idTarget server only rejected 1 mammalian protein, sirtuin 5, which confirms the reliability of the molecular similarity comparison. It is also remarkable that all known complexes for quercetin available in the PDB at the time of the assay were retrieved.

According to the docking poses obtained by idTarget, quercetin reaches a known binding site of every protein which is generally a catalytic or an allosteric site (Fig. 2). While idTarget accurately predicted protein binding sites, docking poses did not always resemble the co-crystallized ligand orientation. Frequently quercetin appeared in the same plane but with opposite orientation. This divergence can be explained by the quercetin inner symmetry. Additionally, a small number of docking simulations showed peculiarities, for example a few sirtuin 3 docking poses overlapped with the protein substrate, glycogen phosphorylase docking poses were located in a known allosteric site normally occupied by the endogenous ligand AMP, sulfotransferases and ATPase docking poses could also bind the ATP site proximal to an allosteric site. Except for sulfotransferases all these proteins showed unfavorable Z-scores and predicted affinities. Proteins in

this latter group have wide and exposed binding sites in contrast with the best scoring candidates. According to idTarget score and taking into account the qualitative considerations expressed above, proteins such as macrophage migration inhibitory factor, troponin C, transthyretin, and ATP synthase among others should not be prioritized as target candidates.

An interesting finding of the present study is that most of the endogenous ligands, substrates or cofactors related to the resulting protein set are nucleotide derivatives like ATP/ADP, FAD, NAD, pyridoxal phosphate, PAPS, PAP, HIBYL-CoA and xanthine. The protein target candidates greatly vary in sequence length, fold and quaternary structure, meaning that any structural similarity found among them might be rather local than global. In the first instance, structural similarity between quercetin and ATP/ADP was thus analyzed. The result of this analysis (Fig. 3) indicated that quercetin may work as an ATP mimetic. In this regard, previous studies suggested a correspondence between the benzopyranone moiety of the flavonoid and the adenine part of the ATP as well as the ortho-dihydroxyphenyl and the phosphates of ATP (Teillet et al., 2007).

Additionally, a comparison between idTarget score and MOE score is depicted in Fig. 4 showing a rough but significant agreement between both systems (p-value < 0.0001) supporting idTarget screening. MOE docking incorporates GBVI/WSA dG, a forcefield-based scoring function which estimates the free energy of binding of the ligand from a given pose as well as the solvation effect and allows the explicit representation of co-factors and substrates in the vicinity of the binding site which supposed an improvement of idTarget results.

3.2 Molecular dynamics simulations of quercetin-target candidate complexes

Taking into consideration their biological relevance we evaluated a selected group of protein candidates by means of molecular dynamics. This is a more realistic approach that allows the dynamic behavior of a system and the explicit influence of solvent molecules to be addressed as would occur in a living organism.

The following quercetin-protein complexes were generated: glycogen synthase kinase-3 beta (GSK-

3b) from 1j1b crystal, mitogen-activated protein kinase 14 (MAPK14) from 3s3i crystal, phosphatidylinositol 3-kinase gamma (PI3K) from 1e8w crystal, and poly [ADP-ribose] polymerase 1 (PARP) from 3gjw crystal. For MAPK14 a different crystal was chosen from the one identified in the screening due to a missing loop in the structure that could not be correctly completed in MOE. Three 10 ns simulations were run for each system in a periodic water box at 300 K. Ramachandran plots indicated that phi/psi dihedral angles of each protein chain generally remained under allowed regions with few exceptions located nearby. Each system showed an initial divergence from the starting point of around 1 Å measured at backbone atoms (see Table 5 from supplementary material). Frames along the simulation had a divergence of around 0.5 (not shown). Total potential energy and interaction potential energy measured along the simulation indicated a homogeneous behavior of the systems and showed that quercetin was stabilized in the protein binding site (see Fig. 1 to 3 from supplementary material). The average values of three 10 ns simulations measured from the last ns were -98,4 kcal for GSK-3b, -112,3 kcal for MAPK14, -123,1 kcal for PI3K and -113,8 kcal for PARP1. A characteristic interaction pattern was observed involving residues with hydrogen donating or accepting moieties like glutamate, aspartate and serine (Fig. 5). It was the receptor protein which contributed with most of the interaction energy through hydrogen-donating and accepting groups and to a lesser extent aromatic residues and also showed an explicit involvement of water molecules in the binding. Solvent contribution with the interaction energy grows from around 15 % in the case of PI3K, 30 % in PARP1 and MAPK14 complexes and 40 % in GSK-3b. Another major feature of quercetin interaction is the participation of the different hydroxyl groups, being substitutions in positions 3', 4' and 7 those which were generally involved in direct contacts with the target. Interestingly these feature are shared by other polyphenols like resveratrol with similar biological effects documented.

3.3 Functional characterization

The ranked candidate set comprises proteins with a broad range of macromolecular folds and

biological activities including enzymes, transcription factors and transporters, and can be divided into proteins with direct metabolic action like xanthine oxidase and proteins involved in intracellular signaling pathways. The significantly enriched gene ontology (GO) functional annotations of quercetin target candidates include regulation of cell death and proliferation and KEGG pathways highlight their involvement in cancer and neurodegeneration (annotations incorporated in Table 1). The role of some target proteins in neuronal physiology is also notable, given their involvement in neurotrophin signaling, neuronal differentiation and synapse function (not shown). These correspond with already reported effects of flavonoids (Blasina et al., 2009; Chen et al., 2015; Spencer, 2009). STRING protein-protein interaction network analysis illustrates many functional associations among the target candidates supporting their participation in related biological processes (Fig. 6).

Remarkably, there is a wide array of predicted proteins like tyrosine protein kinase Src, casein kinase 2 and vascular endothelial growth factor receptor 2 which have been tested by experimental assays supporting their interaction with quercetin and/or related flavonoids like myricetin, apigenin and luteolin (Table 2). Furthermore, among the predicted candidates, experimentally tested and untested proteins behaved equally in terms of docking scores having no statistically significant difference (t-test). On the whole the predicted target candidates are associated with a spectrum of biological activities that is in agreement with the multi-target mechanism of action proposed for quercetin (Table 1). Considering the underlying structural similarities, being kinases and PARPs the two main protein folds involved, it is safe to assume the predicted candidates constitute a whole group of quercetin protein targets. Predicted candidates that have not previously been reported could be of interest for drug discovery and future target validation.

Protein kinases should be highlighted, a number of which have been recognized as promising drug targets for tumor therapy and neurodegeneration including MAP kinases, receptor tyrosine kinases, and kinases related to the PI3-kinase/Akt/mTOR signaling pathway (Han et al., 2015; Sebolt-

Leopold and Herrera, 2004; Zhang et al., 2015; Zhou and Huang, 2012). For example, p38 MAPK is a key protein kinase involved in neuronal apoptosis and inflammation, whose inhibition has been proposed as a treatment against ischemic damage and neurodegeneration (Zhang et al., 2015). In this regard, previous studies have shown that quercetin treatment leads to pro-apoptotic effects in cancer models through modulation of these kinase pathways (Russo et al., 2014). Thus, our results are in agreement with such proposed quercetin effect.

Another interesting group is that of Tankyrases, which are a particular group of poly(ADP-ribosyl)transferases that differ by their overall domain structure and functions. It has been suggested that tankyrase inhibitors could improve anti-cancer effects if combined with other kinase inhibitors such as MEK, epidermal growth factor receptor or PI3-kinase inhibitors in cancer treatment (Lehtiö et al., 2013). PARP-1 is also related to kinase signaling. PARP-1 facilitates diverse inflammatory responses by promoting inflammation-relevant gene expression through NF-kappaB and promotes mitochondria-associated cell death in injured tissues (Ba and Garg, 2011). It is widely accepted that PARP-1 signals to the MAPK pathway by modulating the phosphorylation of ERK1/2, p38, and c-Jun NH2-terminal kinase. Thus far, studies on the relationship between PARP-1 and MAPK suggest that they might stimulate each other in a positive feedback cycle to propagate the responses to long-lasting stress signals (Ba and Garg, 2011). Such crosstalk between protein targets reinforces the concept of multilevel and synergistic mechanisms of action underlying the biological effects of flavonoids.

Another relevant point is the pleiotropism of many of the protein targets in both cancer and neuroprotection. Glycogen synthase kinase 3b is a perfect example of a promising target candidate which is involved in cancer survival and also promotes nuclear export and degradation of Nrf2 (Nuclear Factor-erythroid 2 (NF-E2) p45-related Factor-2), a transcription factor that triggers antioxidant and cytoprotective responses (Jain and Jaiswal, 2007; Russo et al., 2014; Yoshino and Ishioka, 2015).

The metabolism of polyphenols is also suggested by the presence of oxidoreductases and sulfotransferases like sulfotransferase 1B1 and NRH dehydrogenase [quinone] 2 (NQO2). The latter, apparently serves as a quinone reductase in connection with conjugation reactions of hydroquinones involved in detoxification pathways. NQO2 inhibition by quercetin (experimental Kd = 50 nM) resveratrol (Kd = 30 nM) and other polyphenols was suggested to have a role in the induction of antioxidant defenses that could underly their long term chemopreventive effects (Buryanovskyy et al., 2004). Whether this enzyme activity is simply limited to polyphenol excretion or also to the production of biologically active molecules is currently unclear.

Thereis remarkable similarity between quercetin, resveratrol and other flavonoids which share common pharmacophoric features. In accordance with the underlying hypothesis of this work, the target profile of quercetin may not be specific and could be generalized. For instance docking scores between target candidates and related flavonoids extracted from PubChem with a Tanimoto similarity of 98 % like myricetin and fisetin showed in general a difference below 5 % from quercetin.

Flavonoids have been widely studied for their *in vitro* free radical scavenging activity considering it a property that could prevent or delay the onset of oxidative stress related diseases. However, this mechanism of action presents many difficultiessuch as kinetic limitations and the fact that many endogenous antioxidants are present at much higher concentrations. In thisstudy we shed light, in an unbiased way, on the potential protein targets of quercetin that could underlie its wide ranging biological effects. In light of these considerations and the known involvement of protein targets like kinases and PARPs in disease, the polypharmacological profile of quercetin and related flavonoids could be very relevant reinforcing their therapeutic potential.

Even though most multi-target drugs have been discovered serendipitously, rationally designed multi-target directed drugs have shown promising results (Bolognesi and Cavalli, 2016). Indeed, in addition to quercetin described here, several drugs are known to modulate multiple targets, and this

polypharmacological property could underlie therapeutic efficacy (Talevi, 2015). Another potentially favorable feature is that a weak-binding molecule acting on multiple targets can potentially deliver the same outcome as a high-affinity drug acting on a single target (Wang et al., 2016). Moreover, promiscuous, or multi-target drugs, have a higher likelihood of successfully modulating whole cellular networks in diseases driven by multiple pathological processessuch as cancer and neurodegeneration. Despite this, and even though dietary derived compounds are generally recognized as safe, it must be considered that different target profiles could lead to positive or negative effects depending largely on drug dose. How to fine-tune different antagonistic effects for maximal efficacy is an important and emerging area of research (Prati et al., 2016; Wang et al., 2016).

4. Conclusions

In conclusion, in this study, a hierarchical inverse screening approach using SHAFTS-LIBRA and idTarget was employed to identify potential protein targets of quercetin. The screening successfully retrieved every quercetin target contained in the PDB including phosphatidylinositol 3-kinase, xanthine oxidase and expanded the list to new putative quercetin targets like poly(ADP-ribose) polymerases. Most of the predicted panel of targets fall under recognized clinical targets with anti-tumour and neuro-protective effects or target enzymes of drug design such as glycogen synthase kinase-3 beta and mitogen-activated protein kinase 14, and some of them have been well established in experimental settings. In addition, some of these protein targets are involved at different stages within the same signaling pathways or in interconnected signaling pathways, supporting a pleiotropic, multilevel and synergistic mechanism of action of quercetin. Above all the predicted targets could be grouped in two main groups comprised of protein kinases and PARPs. These protein groups established similar interactions with quercetin that persisted during molecular dynamics simulations. These results support the hypotheses that quercetin is a multi-kinase and multi-PARP inhibitor that could act synergistically. PARP-1 as a stress sensor is a potential

therapeutic target in pathologies like cancer, inflammation related diseases and neurodegenerative diseases (Martire et al., 2015). Indeed, multi-kinase inhibitors are a promising therapeutic approach for many pathologies like cancer and viral infections, with imatinib, desatinib and staurosporine as examples (Broekman, 2011; Carra et al., 2013; Kruse et al., 2011; Martinez-Gil et al., 2013). Above all, the interactions described hereconstitute a potential explanation for the multiple and diverse biological effects documented by epidemiological, *in-vivo* and *in-vitro* studies of quercetin and related flavonoids.

However, some issues still need to be addressed. Apart from docking scoring limitations in protein-target ranking, the under representation of rare proteins in the PDB or the absence of proteins for which three-dimensional structures are not available can cause false negatives (Warren et al., 2006; Xie and Bourne, 2005). In addition, to further refine these results other interaction repositories like DrugBank could be incorporated. Additional validation of the results for some target candidates can also be achieved by explicitly using docking benchmark data-sets (decoys) (Bauer et al., 2013; Huang et al., 2006). Moreover, the interactions of more ligand-protein complexes could also be analyzed by means of molecular dynamics simulations to ensure that flexibility of proteins and solvent effects are considered.

In conclusion, reverse screening approaches describes a different workflow to provide valuable information for future *in vitro* and in *vivo* studies with flavonoids or, in general, for drug discovery. Overall, the present results may broaden the understanding of the mechanisms of action of quercetin and generate new hypotheses about its therapeutic potential, allowing a repositioning of quercetin and quercetin analogues for potential future therapeutic developments.

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Tables

Table 1

Disease association and relevant biological process for the target candidates found in the Protein Data Bank (PDB).

Uniprot id	Name	Associated disease	Biological process	Class
		Homo sapiens		
Q00534	Cyclin dependent kinase 6	Cancer		Cell cycle/transferase
Q9H2K2	Tankyrase-2			Transferase/transferase inhibitor
P49888	Estrogen sulfotransferase	Cancer		Transferase
O75897	Sulfotransferase 1C4			Transferase
O00338	Sulfotransferase 1C2	Cancer		Transferase
P50225	Sulfotransferase 1A1	Cancer		Transferase
O43704	Sulfotransferase family cytosolic 1B member 1			Transferase
P09960	Leukotriene A-4 hydrolase	Immune diseases		Hydrolase
P49759	Dual specificity protein kinase CLK1		Proliferation	Transferase
P0DMM9	Sulfotransferase 1A3	Cancer		Transferase
P35968	Vascular endothelial growth factor receptor 2	Cancer	Cell death/proliferation	Transferase/transferase inhibitor
P16083	NRH dehydrogenase [quinone] 2	Cancer, Parkinson's disease		Oxidoreductase
Q13554	Calcium/calmodulin-dependent protein kinase type 2 beta	Cancer	Cell death/neuron related	Transferase
O95271	Tankyrase-1			Transferase/transferase inhibitor
Q6IMI6	Sulfotransferase 1C3			Transferase
Q13627	Dual specificity tyrosine-phosphorylation-regulated kinase 1A			Transferase
Q460N3	Poly [ADP-ribose] polymerase 15			Transferase
Q460N5	Poly [ADP-ribose] polymerase 14			Transferase
P09874	Poly [ADP-ribose] polymerase 1	Alzheimer's disease		Transferase/transferase inhibitor
O60674	Tyrosine-protein kinase JAK2	Cancer	Cell death/proliferation/neuron related	Transferase/transferase inhibitor
P68400	Casein kinase 2 subunit alpha	Parkinson's disease		Transferase
Q9NZL9	Methionine adenosyltransferase 2			Oxidoreductase

	subunit beta			
P15056	Serine/threonine-protein kinase B-raf	Cancer	Cell death/proliferation/neuron related	Transferase/transferase inhibitor
Q9UGN5	Poly [ADP-ribose] polymerase 2		Cell death	Transferase
O14965	Aurora kinase A	Cancer	Cell death/neuron related	Transferase/transferase inhibitor
P23458	Tyrosine-protein kinase JAK1	Cancer	Proliferation	Transferase/transferase inhibitor
O43293	Death-associated protein kinase 3	Cancer	Cell death/neuron related	Transferase
P11362	Fibroblast growth factor receptor 1	Cancer	Cell death/proliferation/neuron related	Transferase/transferase inhibitor
Q96RR4	Calcium/calmodulin-dependent protein kinase kinase 2	Anxiety disorder		Transferase/transferase inhibitor
P49841	Glycogen synthase kinase-3 beta	Alzheimer's disease, cancer	Cell death/neuron related	Transferase
O94768	**Serine/threonine-protein kinase 17B**		Cell death	Transferase
O14757	Serine/threonine-protein kinase Chk1	Cancer	Proliferation	Transferase
P24941	Cell division protein kinase 2	Cancer		Transferase
P51813	Cytoplasmic tyrosine-protein kinase BMX		Cell death/proliferation	Transferase/transferase inhibitor
P52333	Tyrosine-protein kinase JAK3		Cell death/proliferation	Transferase
Q16539	Mitogen-activated protein kinase 14	Parkinson's disease, Amyotrophic Lateral Sclerosis	Cell death, neuron related	Transferase/transferase inhibitor
P45983	Mitogen-activated protein kinase 8	Cancer, Parkinson's disease	Cell death, neuron related	Transferase/transferase inhibitor
P08631	**Hematopoetic cell kinase HCK**	Immune diseases, Parkinson's disease	Cell death	Transferase
P12931	Proto-oncogene tyrosine-protein kinase Src	Cancer	Cell death/proliferation/neuron related	Tyrosine-protein kinase
P50750	Cell division protein kinase 9		Proliferation	Transferase
O14936	Peripheral plasma membrane protein CASK	Microcephaly	Proliferation	Transferase
P37231	Peroxisome proliferator-activated receptor gamma	Diabetes, Cancer, Alzheimer's disease, Huntington's disease	Cell death	Transcription regulation
Q9Y6E0	Serine/threonine-protein kinase 24		Cell death/neuron related	Transferase
P21802	Fibroblast growth factor receptor 2	Cancer	Cell death/proliferation/neuron related	Transferase
P11309	**Serine/threonine-protein kinase**		Cell death	Transferase

	PIM-1			
O15530	Phosphoinositide-dependent kinase-1	Cancer	Cell death/neuron related	Transferase
P00519	Tyrosine-protein kinase ABL1	Cancer	Cell death/proliferation/neuron related	Transferase
P06239	Tyrosine-protein kinase LCK	Diabetes	Cell death/proliferation/neuron related	Transferase
Q08881	Tyrosine-protein kinase ITK/TSK	Immune diseases	Proliferation	Transferase/transferase inhibitor
Q9H0J9	Poly [ADP-ribose] polymerase 12			Transferase
P45984	Mitogen-activated protein kinase 9	Cancer	Cell death/neuron related	Transferase
Q8N5Y8	Poly [ADP-ribose] polymerase 16		Cell death	Transferase/transferase inhibitor
Q6NVY1	**3-Hydroxyisobutyryl-CoA hydrolase**			Hydrolase
P29320	Ephrin type-A receptor 3		Neuron related	Transferase
P14174	Macrophage migration inhibitory factor	Immune diseaases, Alzheimer's disease	Cell death	Isomerase
P63316	Troponin C, slow skeletal and cardiac muscles		Cell death	Contractile protein
Q13464	Rho-associated protein kinase 1	Cancer	Cell death/neuron related	Transferase
Q99683	Mitogen-activated protein kinase kinase kinase 5	Amyotrophic Lateral Sclerosis	Cell death/neuron related	Transferase/transferase inhibitor
P49137	MAP kinase-activated protein kinase 2		Neuron related	Transferase
P53779	Mitogen-activated protein kinase 10	Epileptic encephalopathy	Neuron related	Transferase/transferase inhibitor
O00311	Cell division cycle 7-related protein kinase		Proliferation	Transferase
P04746	Pancreatic alpha-amylase	Alzheimer's disease, Parkinson's disease		Hydrolase/hydrolase inhibitor
P02766	Transthyretin	Amyloidosis		Transport protein
Q9Y6F1	Poly [ADP-ribose] polymerase 3	Cancer		Transferase/transferase inhibitor
Q9NTG7	NAD-dependent protein deacetylase Sirtuin-3	Aging		Hydrolase/hydrolase inhibitor
Q53GL7	Poly [ADP-ribose] polymerase 10		Proliferation	Transferase
	Mus musculus			
P54763 (P29323)	Ephrin type-B receptor 2	Cancer		Transferase

Rattus norvegicus				
P63086 (P28482)	Mitogen-activated protein kinase 1	Alzheimer's disease, cancer	Cell death/proliferation/neuron related	Transferase
Oryctolagus cuniculus				
P00489 (P11217)	Glycogen phosphorylase, muscle form			Transferase/transferase inhibitor
Sus scrofa				
O02697 (P48736)	**Phosphatidylinositol 3-kinase catalytic subunit**	Aging, Cancer	Cell death, neuron related	Phosphoinositide 3-kinase gamma
Bos taurus				
P80457 (P47989)	**Xanthine dehydrogenase/oxidase**		Cell death	Oxidoreductase
Q28021 (O75116)	Rho-associated protein kinase 2	Cancer	Cell death/neuron related	Transferase
P19483 (P25705)	**ATP synthase**	Alzheimer's disease, Parkinson's disease, Huntington's disease		Hydrolase

Candidate proteins are segregated by source organism and depicted according to the corresponding Uniprot entry (human homologue in parenthesis), name, PDB class, related disease and biological process. Proteins co-crystallized with quercetin are depicted in bold. The information was gathered from publicly available databases like PDB, Uniprot and gene ontology repositories (Berman, 2000; Consortium, 2014; Dennis et al., 2003).

Table 2

Experimental assays supporting quercetin target candidates.

Name	PubChem BioAssay AID; PDB	Literature
Cyclin dependent kinase 6	242481, 109522, 1095229, 1224758	
Tankyrase-2	735784 (apigenin, luteolin)	
Estrogen sulfotransferase	747	
Dual specificity protein kinase CLK1	382, 1224761	Reference (Boly et al., 2011)
Vascular endothelial growth factor receptor 2	1063038	Reference (Pratheeshkumar et al., 2012)
NRH dehydrogenase [quinone] 2		Reference (Buryanovskyy et al., 2004)
Calcium/calmodulin-dependent protein kinase type 2 beta	673630, 353599, 1224752	Reference (Boly et al., 2011)
Tankyrase-1	735786 (apigenin, luteolin)	
Dual specificity tyrosine-phosphorylation-regulated kinase 1A	588345 (myricetin), 504441 (kaempferol, luteolin), 588345 (keampferol)	
Poly [ADP-ribose] polymerase 1	735783 (apigenin, luteolin)	
Tyrosine-protein kinase JAK2		Reference (Boly et al., 2011)(Luo et al., 2016)
Casein kinase 2 subunit alpha	353598, 378676, 435640, 587344	Reference (Lolli et al., 2012)
Aurora kinase A		Reference (Boly et al., 2011)
Tyrosine-protein kinase JAK1	1224770	Reference (Boly et al., 2011)(Kumamoto et al., 2009)
Death-associated protein kinase 3	1224767	
Fibroblast growth factor receptor 1	378675 (apigenin)	
Calcium/calmodulin-dependent protein kinase kinase 2	1224756	
Glycogen synthase kinase-3 beta	1115335, 1224769, 512292, 512292	
Serine/threonine-protein kinase 17B	PDB: 3LM5	
Serine/threonine-protein kinase Chk1	512298	
Cell division protein kinase 2	298693, 1224757	
Tyrosine-protein kinase JAK3		Reference (Boly et al., 2011)
Mitogen-activated protein kinase 14	512278, 512278, 603838	
Mitogen-activated protein kinase 8	512277, 603841, 512277	
Hematopoetic cell kinase HCK	PDB: 2HCK	
Proto-oncogene tyrosine-protein kinase Src	420260; 1063039	
Peroxisome proliferator-activated receptor gamma	517390, 743191, 439367, 439368, 439367, 439368 (pathway assays)	
Fibroblast growth factor receptor 2		Reference (Boly et al.,

		2011)
Serine/threonine-protein kinase PIM-1	393, 418378, 706714, 257081, 1063041, 257081, 311148, 257079, 257080, 257082, 1224786, 257079; PDB: 4LMU	Reference (Boly et al., 2011)
Phosphoinositide-dependent kinase-1	1224785; 512288	
Tyrosine-protein kinase ABL1	588664 (myricetin)	Reference (Boly et al., 2011)
Tyrosine-protein kinase LCK	378681, 512297, 512297	
3-Hydroxyisobutyryl-CoA hydrolase	PDB: 3BPT	
Mitogen-activated protein kinase kinase kinase 5	515, 1224773	
MAP kinase-activated protein kinase 2	512283	
Mitogen-activated protein kinase 10	530	
Transthyretin	1239060 (apigenin, luteolin), 1239061 (apigenin, luteolin)	
Mitogen-activated protein kinase 1	512276, 512276	
Phosphatidylinositol 3-kinase catalytic subunit	1185177, 325654, 378677, 1120054; PDB: 1E8W	
Xanthine dehydrogenase/oxidase	1185460, 1185463, 42472, 424718, 424725, 424721, 424722, 424735, 424736, 424727, 424729, 424723, 424734, 424719, 424720, 424724, 424728, 424730, 1238320, 424718, 424723, 424725, 424734; PDB: 3NVY	
Rho-associated protein kinase 2	512293; 512293	
ATP synthase	338025, 338026; PDB: 2JJ2	

Bio-assay information was gathered from *PubChem* and *PubMed* repositories, tested targets are presented along the *PubChem* assay ID and/or associated publication. Protein co-crystallized with quercetin were are also included.

Figures

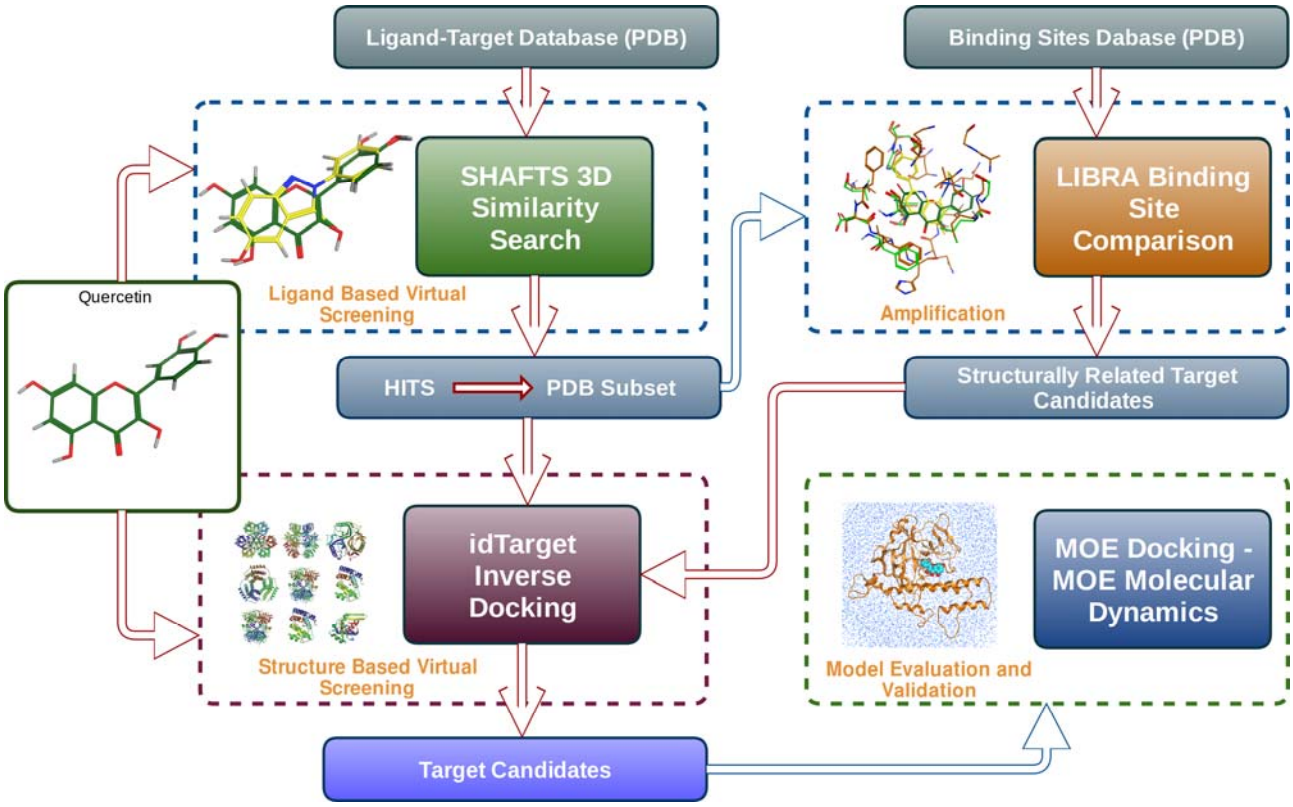

Fig. 1. Reverse screening work-flow. The strategy involved a similarity search with SHAFTS (SHApe-Feature Similarity) algorithm of co-crystalized ligands from the Protein Data Bank (PDB) (Liu et al., 2011). An amplification stage is added to identify related proteins by LIBRA (Ligand Binding site Recognition Application) binding site comparison (Hung et al., 2015). Finally, protein candidates are submited to reverse docking with idTarget web server (*http://idtarget.rcas.sinica.edu.tw/*) (Wang et al., 2012).

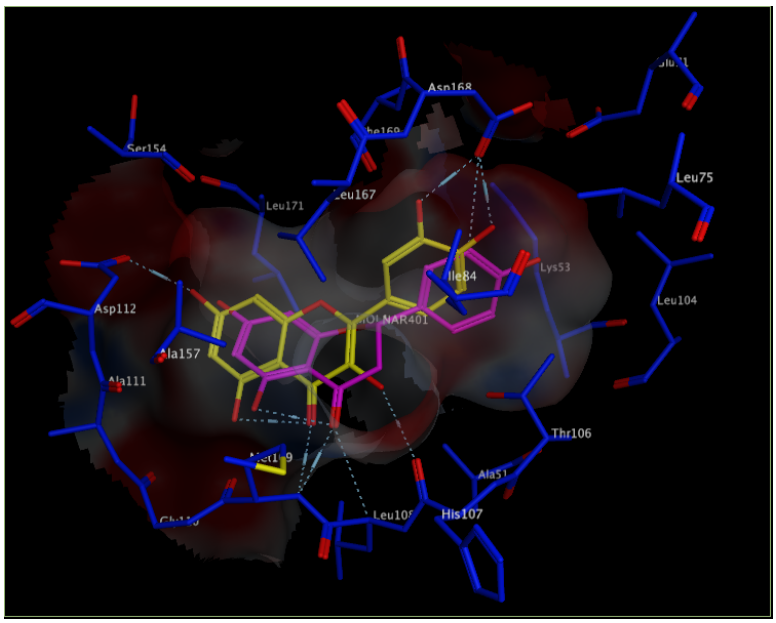

Fig. 2. Result of quercetin (yellow sticks) molecular docking with mitogen-activated protein kinase 14 (p38 MAP kinase) co-crystallized with naringenin (violet sticks) (Protein Data Bank accession id 4eh3) according to idTarget web server (*http://idtarget.rcas.sinica.edu.tw/*) (Wang et al., 2012). A dotted electrostatic surface was drawn around quercetin at a Van der Waals distance, areas depicted in red color correspond to a negative charged surface and white surface to a neutral charge. A good overlapping between naringenin a quercetin docking pose is observed confirming the reliability of the docking method.

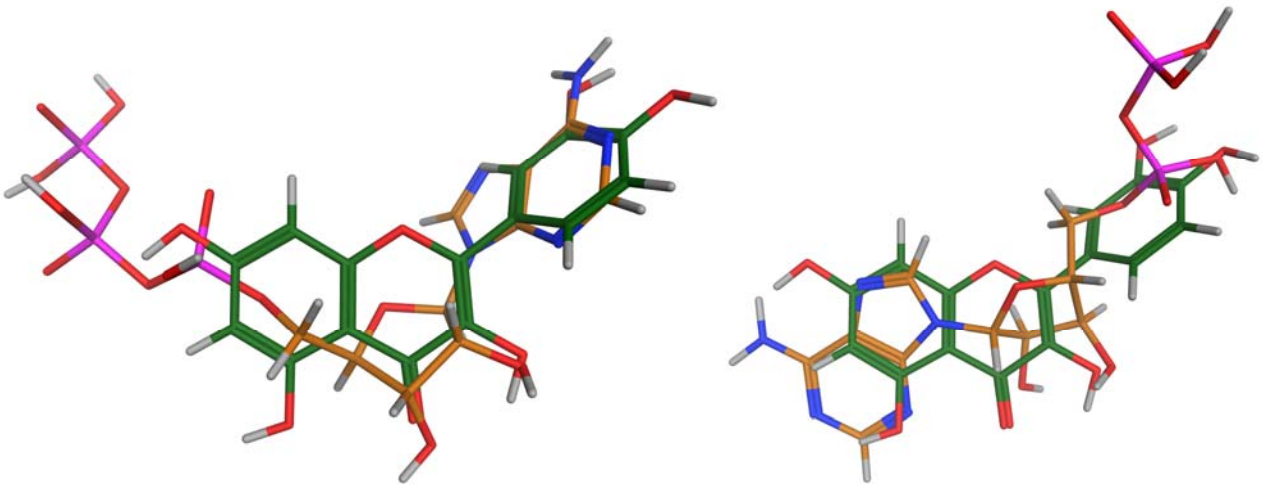

Fig. 3. Structural alignment between ATP/ADP and quercetin given by SHAFTS (SHApe-Feature Similarity) algorithm (Liu et al., 2011). The alignment shows two different quercetin orientations (ATP alignment similarity score: 0.8693, ADP alignment similarity score: 0.9790) that can overlap functional groups of these endogenous ligands and suggests a quercetin ATP/ADP mimetic action.

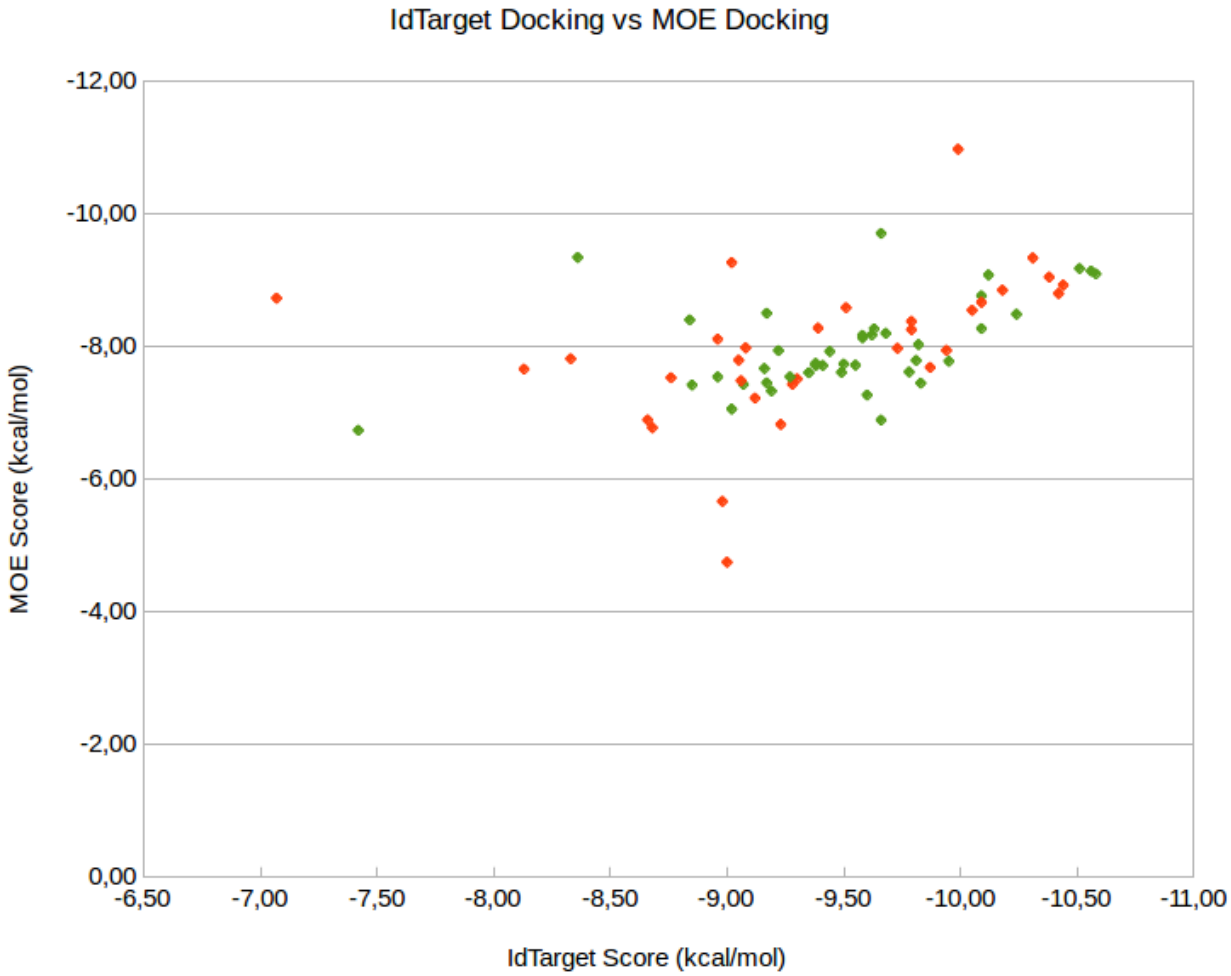

Fig. 4. Comparison between idTarget docking score and MOE docking score. For docking porpoise a single crystallographic structure representative of each protein was used in MOE. The score of the best ranking pose in MOE was compared against the best idTarget score. Orange dots: predicted targets, green dots: predicted and experimentally assayed targets. See Methods for further details.

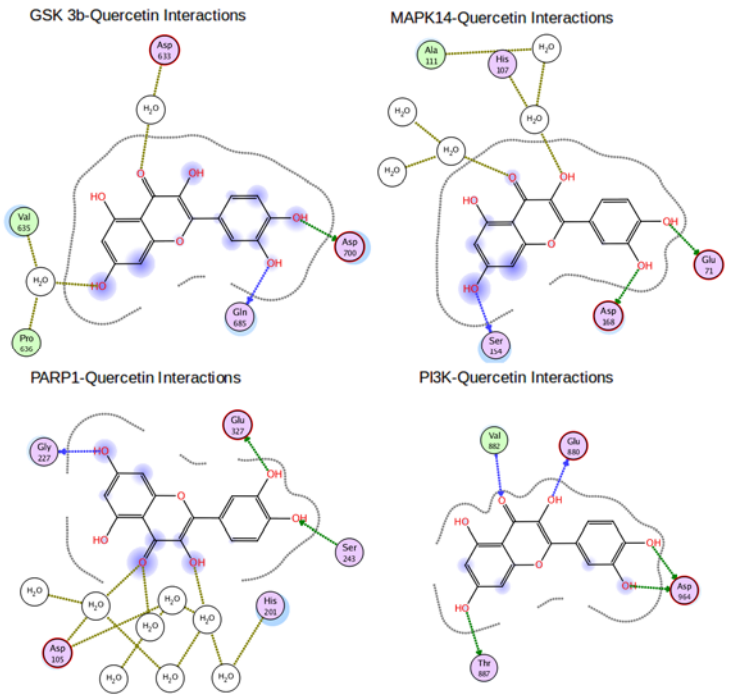

Fig. 5. Protein-ligand interaction depictions of quercetin-target complexes by the end of molecular dynamics simulations (10 ns). Green circles represent greasy, purple circles represent polar, red circles represent acidic and blue circles represent basic amino acids. Protein contacts are depicted by a blue half moon around the amino acids. Blue arrows represent backbone acceptors; green ones depict side chain acceptors and side chain donors. Green benzol rings with a "+" describe an arene–cation binding; 2 benzol rings, an arene–arene binding. Areas with a blue background are exposed to the ligand. The purple dotted lines represent metal contact (Clark and Labute, n.d.).

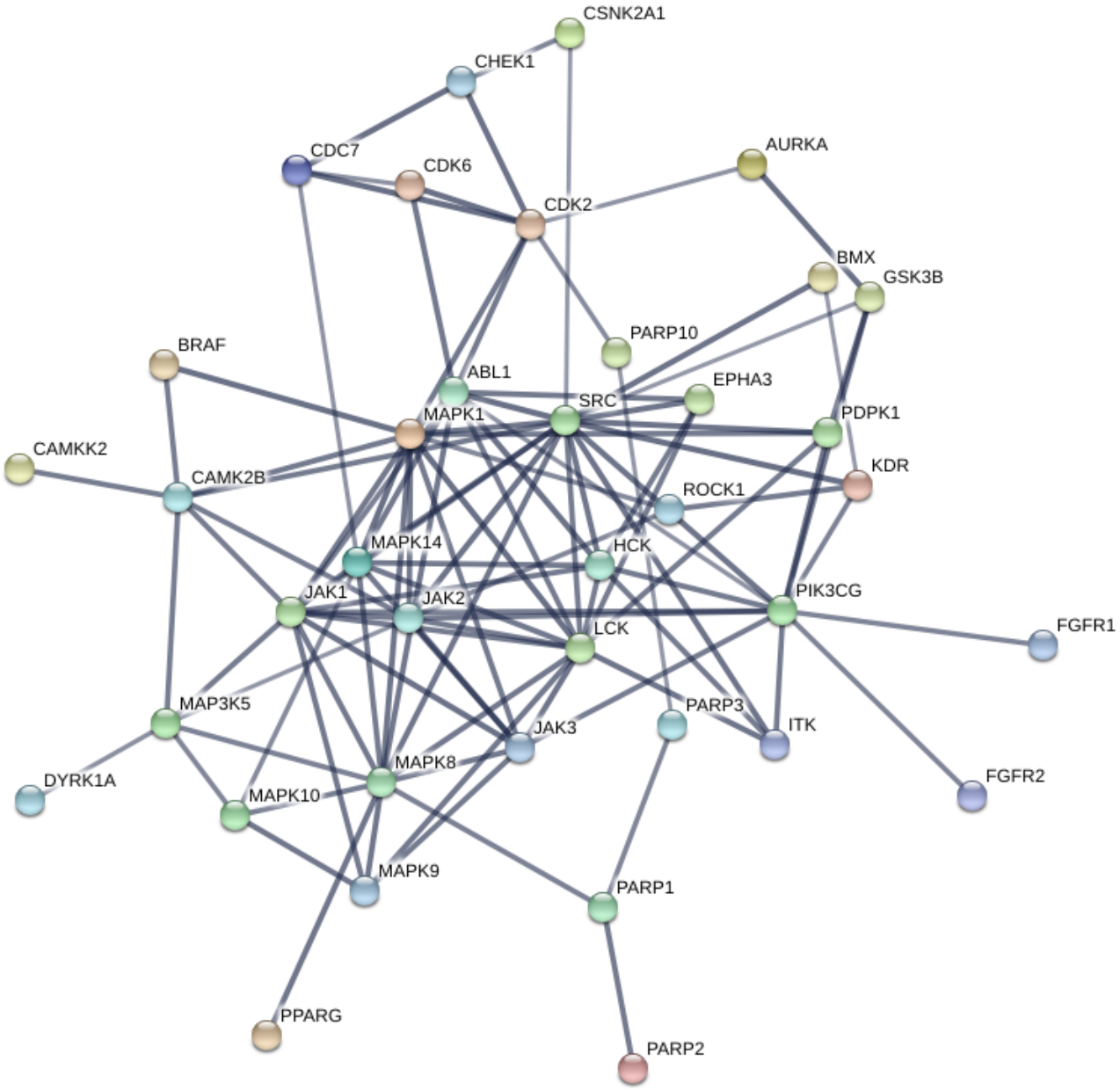

Fig. 6. STRING protein-protein interaction network analysis of target candidates. STRING database establishes functional associations between protein pairs based on documented experimental evidence, homology and text mining inference including protein modification, aggregation, genomic neighborhood and co-expression (Jensen et al., 2009). Only high confidence associations were depicted (0.7 probability and above).

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